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Research Note—

Enhanced Gross Visualization of Chicken Peyer's Patch: Novel Staining Technique Applied to Fresh Tissue Specimens

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SUMMARY. The ileal Peyer's patches (Pp), secondary gut-associated lymphoid tissue of the mucosal immune system, may serve as an important site for monitoring inflammatory and immunologic responses of the host against enteric pathogens. Chicken Pp are often difficult to observe grossly, and a simple technique to enhance visualization of the Pp is lacking. Therefore, we designed a novel staining method that is quick, easy, and accurate to aid in gross identification and recovery of the chicken Pp from fresh tissue specimens. Lower alimentary tracts were harvested from White Leghorn hens and commercial broilers. The ileocecocolic region was excised intact, flushed with deionized water to remove ingesta, and a dilute eosin-Y solution was infused. After 1 min, the eosin-Y was gently extruded. Modified-crystal violet (mCV) was then injected into the gastrointestinal segment, where on the lymphoid tissue area became apparent at the serosal surface. The distal ileal Pp was visible as a pale whitish pink ovoid-focalized area with surrounding gut tissue stained light purple. The exact Pp site could be delineated at the serosal and mucosal surface by gross assessment. Light microscopy evaluation of hematoxylin and eosin-stained tissue slides prepared from the excised Pp site revealed lymphoid tissue aggregations with multiple follicular units indicative of Pp. The novel eosin-Y + mCV staining technique promotes rapid identification and accurate recovery of chicken Pp lymphoid tissue from fresh tissue specimens.

RESUMEN. *Nota de Investigación*—Incremento en la visualización macroscópica de las placas de Peyer en pollos: Novedosa técnica de tinción aplicada a muestras frescas de tejido.

Las placas de Peyer en el íleo, el tejido linfoide secundario asociado al intestino, puede servir como un sitio importante para el seguimiento de las respuestas inflamatorias e inmunológicas del huésped contra patógenos entéricos. Las placas de Peyer en los pollos son generalmente difíciles de observar macroscópicamente y no existe una técnica simple para mejorar la visualización de las mismas. En consecuencia, se diseñó un método de tinción que es rápido, fácil y preciso con la finalidad de ayudar en la identificación macroscópica y la obtención de las placas de Peyer a partir de muestras de tejido fresco de los pollos. Se extrajo el tracto alimentario inferior de gallinas leghorn blancas y pollos de engorde comerciales. Se tomó la región íleocecal completa, se lavó con agua desionizada para remover la ingesta y se le infundió una solución de eosina-Y. Luego de un minuto se retiró la eosina-Y. Después, se inyectó cristal violeta modificado en la superficie de la serosa del segmento gastrointestinal donde el tejido linfoide se hizo evidente. La placa de Peyer de la región distal de íleo se mostró como un área ovoide focalizada de color rosado pálido, rodeada por tejido intestinal teñido de morado claro. La forma exacta de la placa de Peyer se pudo delinear en la superficie de la mucosa y de la serosa mediante evaluación macroscópica. La evaluación al microscopio corriente de las placas de Peyer extraídas y teñidas con hematoxilina y eosina, reveló agregados de tejido linfoide con múltiples unidades foliculares, correspondiendo a las placas de Peyer. La nueva técnica de tinción utilizando eosina-Y más cristal violeta modificado, promueve la identificación rápida y la obtención precisa de tejido linfoide (placas de Peyer) a partir de muestras frescas de tejido en pollos.

Key words: Peyer's patch, eosin-Y, crystal violet, gut-associated lymphoid tissue, mucosal immune system, chicken alimentary tract

Abbreviations: CI = color index; GALT = gut-associated lymphoid tissue; GI = gastrointestinal tract; H&E = hematoxylin and eosin; mCV = modified-crystal violet; PBS = phosphate-buffered saline; Pp = Peyer's patch; sIgA = secretory-immunoglobulin A; SPF = specific pathogen-free

The Peyer's patches (Pp) are classified as secondary or peripheral gut-associated lymphoid tissue (GALT), a component of the extensive, integrated mucosal immune system (2,15). The aggregated lymphoid nodules of a Pp function as lymphoid inductive sites in the alimentary tract (18,22,24). The associated cellular repertoire of the Pp includes macrophages, dendritic cells, plasma cells, and B- and T-lymphocytes (5,14,21,24,29). Antigenic stimulation at Pp can induce effective mucosal and systemic immune responses (7,8,17,21,27). Locally, antigen-specific secretory-immunoglobulin A (sIgA) production can be elicited to afford a protective barrier against infection and invasion by enteric pathogens (6,9,13,16,19,26). Conversely, the Pp may be manipulated by some enteric pathogens to serve as a portal of entry (4,10,11,20,25,28). Therefore, the Pp may be a pertinent site for research interests regarding enhancement of host mucosal immunity and disease pathogenesis studies.

The chicken (*Gallus domesticus*) seems to exhibit a conserved locality of Pp lymphoid tissue, among different age groups, within the region of the distal ileum (1,3). Kajiwara *et al.* (12) performed immunohistochemical staining of the embryonic intestine that demonstrated the development of a distal ileal Pp cranial to the ileocecal junction and one other Pp site in proximity to the Meckel's vitelline diverticulum at days E13–E15 of chick embryogenesis (12). Histological studies conducted by Befus *et al.* (1) yielded evidence of distal ileal Pp lymphoid tissue that persisted in outbred, White Leghorn chickens ranging from 1.5 weeks, 6–12 weeks, 16–36 weeks, and 52–58 weeks of age. This one persistent Pp may therefore serve as an important lymphoid tissue site for monitoring inflammatory changes or immunologic responses of the avian host

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against enteric pathogens. It also might have merit in age-related comparison studies of the immune system.

However, on cursory gross inspection of the serosal surface of the alimentary tract, the precise location of the distal ileal Pp of chickens can often be difficult or impossible to determine. The chicken Pp are not as prominent in gross morphological appearance compared with the nodular Pp structure of mammalian species such as mice and rabbits. The GALT in some chickens may regress or undergo involution, becoming even less distinct as those chickens age (1). The lack of a simple technique to enhance the Pp in fresh tissue specimens has impeded the accurate and speedy recovery of such lymphoid tissue from poultry. We developed a novel staining method, which enhanced gross visualization of the chicken Pp *in situ*. The staining technique was applicable for assessment of Pp areas *via* serosal or mucosal surface and for the subsequent precise excision of Pp lymphoid tissue.

MATERIALS AND METHODS

Chickens. Four commercial broilers 4–6 weeks of age were received dead from a local commercial poultry processing facility. Seventeen White Leghorn chickens 70+ weeks of age were obtained from the specific-pathogen-free (SPF) stock maintained at the USDA, Southeast Poultry Research Laboratory, Athens, GA. The SPF White Leghorn chickens were euthanitized by CO_2 gas inhalation, in accordance with guidelines established by USDA, Institutional Animal Care and Use Committee.

Postmortem tissue collection. Upon receipt of the commercial broilers and briefly after euthanasia of SPF hens, the lower gastrointestinal (GI) tracts were carefully dissected intact from the postmortem chickens. Debridement of the intestinal mesenteric attachments was performed so that the intestine could be extended full length. A portion from each intestinal tract was excised by making a proximal incision near the jejunoileal junction (Meckel's diverticulum) and a distal incision of the colon–cloaca region. The resected segment, proximal ileum extending distally to colon, was then flushed with 40–60 ml of room temperature deionized water to remove excess ingesta and any cellular debris from the intestinal lumen. The staining procedure should be conducted immediately subsequent to GI segment flushing.

If the staining procedure cannot be carried out directly, the flushed segments should be placed in Whirl-Pak bags (Fisher Scientific Co., Suwanee, GA) filled with room temperature phosphate-buffered saline (PBS) to prevent tissue desiccation. Segments may then be stored temporarily at 6–8 C for no more than 30–60 min before implementation of staining.

Preparation of stains. The staining method used two common stains: the anionic dye eosin-Y (color index no. 45380) and the cationic dye crystal violet (CI number 42555). The ready-made stain formulation of eosin-Y, Hema-3 Solution I (Fisher Scientific Co.), was diluted as 12.5 ml per 250 ml of PBS, pH 7.4 (P-3813; Sigma, St. Louis, MO). A modified crystal violet (mCV) stock solution was prepared as follows: 2 g of crystal violet powder (C-0775; Sigma), 20 ml of 95% ethyl alcohol, and 100 ml of deionized water. The mCV stain formulation contained no ammonium oxalate. The mCV stock solution was further diluted as 250 µl per 250 ml of PBS to obtain a working concentration.

Intestinal staining procedure. The flushed intestinal segment was ligated at the proximal ileal end (jejunoileal junction) by using Carmalt forceps. First, the diluted eosin-Y solution was infused into the gastrointestinal tract segment by using a 20–30-cc syringe, 14–16 gauge 1-in. needle. The needle was threaded into the intestinal lumen at the ileocecal junction, and a volume of approximately 10–20 ml of eosin-Y stain solution was injected to achieve full distention of the intestinal segment. The eosin-Y was allowed to permeate for 1 min, and the stain solution was then gently extruded. The diluted mCV stain solution was then injected into the intestinal lumen at a volume of approximately 10–20 ml to obtain gut distention. The serosal surface was immediately/directly assessed for any grossly apparent color variability, indicative of Pp tissue, which ensued subsequent to infusion of the mCV.

It was imperative that the mCV was not sequestered/retained within the intestinal lumen for an extended time, because a generalized purple staining effect of GI tissue will occur with prolonged exposure to CV dye. If the presumptive Pp site was not visualized immediately after injection of the mCV solution, then the mCV was extruded from the lumen within 60 sec, and the intestinal segment was redistended with PBS to allow for further evaluation without risk of overstaining.

Poststaining gross evaluation of intestine for presumptive Pp sites. The alimentary tract segments were evaluated grossly from both the serosal and mucosal surfaces for defined areas that exhibited red–pink versus purple color variation. A suspect/presumptive Pp site identified from the serosal surface was marked at its periphery with a mounting-pin or tissue marking dye (Fisher Scientific Co.) to tag the specific location. The intestine was then cut longitudinally along the mesenteric border and reflected to expose the mucosal/luminal surface. The mucosa was examined by the naked eye for areas delineated by contrasts in stain coloration and structural/morphological composition.

Microscopic evaluation of presumptive Pp sites. The presumptive Pp sites were excised using iris scissors or a 6-mm biopsy punch (Fray Products Corp., Buffalo, NY) and then fixed for 24 hr in 10% buffered formalin. The fixed tissues were routinely processed, paraffin-embedded, sectioned, and hematoxylin and eosin (H&E) stained. The H&E tissue slides were then examined via bright-field microscopy. Microscopic evaluation was performed to determine lymphoid tissue presence or absence and to assess sites for morphologic characteristics indicative of Pp.

RESULTS

Gross observations. The distal ileal Pp site was enhanced in fresh tissue alimentary tract segments from commercial broilers and SPF White Leghorn hens by infusion of eosin-Y (Fig. 1A) followed by mCV (Fig. 1B) stain solution. In conjunction with our observation of a distal ileal Pp, we also were able to identify a second consistent focalized Pp site along the proximal ileal region near the Meckel's diverticulum, with the application of our staining technique (Fig. 1B).

Pp location, as observed grossly from the serosa, was visualized by the emergence of a pale pink or white focalized area with the surrounding gut tissue diffusely stained a contrasting light purple (Fig. 1C). When assessed from the mucosal aspect, the Pp presented as a pink focal area circumscribed by a dark purple marginal zone with radiating diffuse light purple coloration of the peripheral mucosa/gut epithelia (Fig. 1D). The distal ileal Pp was located approximately 7–10 cm cranial to the ileocecal junction and was circular/ovoid. The proximal ileal Pp site was positioned 3–6 cm caudal to the Meckel's diverticulum and was slightly more ellipsoidal. Longitudinal lengthwise measurements of these pale staining ovoid/ellipsoid areas were in the 0.7–2.0-cm-diameter size range. Based on the color variation, the presumptive Pp tissue sites could be delineated and then be easily dissected from the intestinal segment.

Microscopic observations. The eosin-Y + mCV coloration observed in fresh tissue specimens was not retained through routine fixation and histologic processing steps. Interference with H&E staining of the formalin-fixed, paraffin-embedded tissue sections was not detected. Structural interpretations of H&E stained tissue slides via light microscopy were performed as usual.

Light microscopy assessment of the excised tissue sites yielded characteristics representative of ileal Pp. The evaluation of the H&E-stained slides revealed a substantial proportion of organized lymphoid tissue present in the presumptive Pp areas. Aggregations of lymphoid tissue were evident in the lamina propria (Fig. 2A). Multiple lymphoid follicles and diffuse interfollicular lymphoid

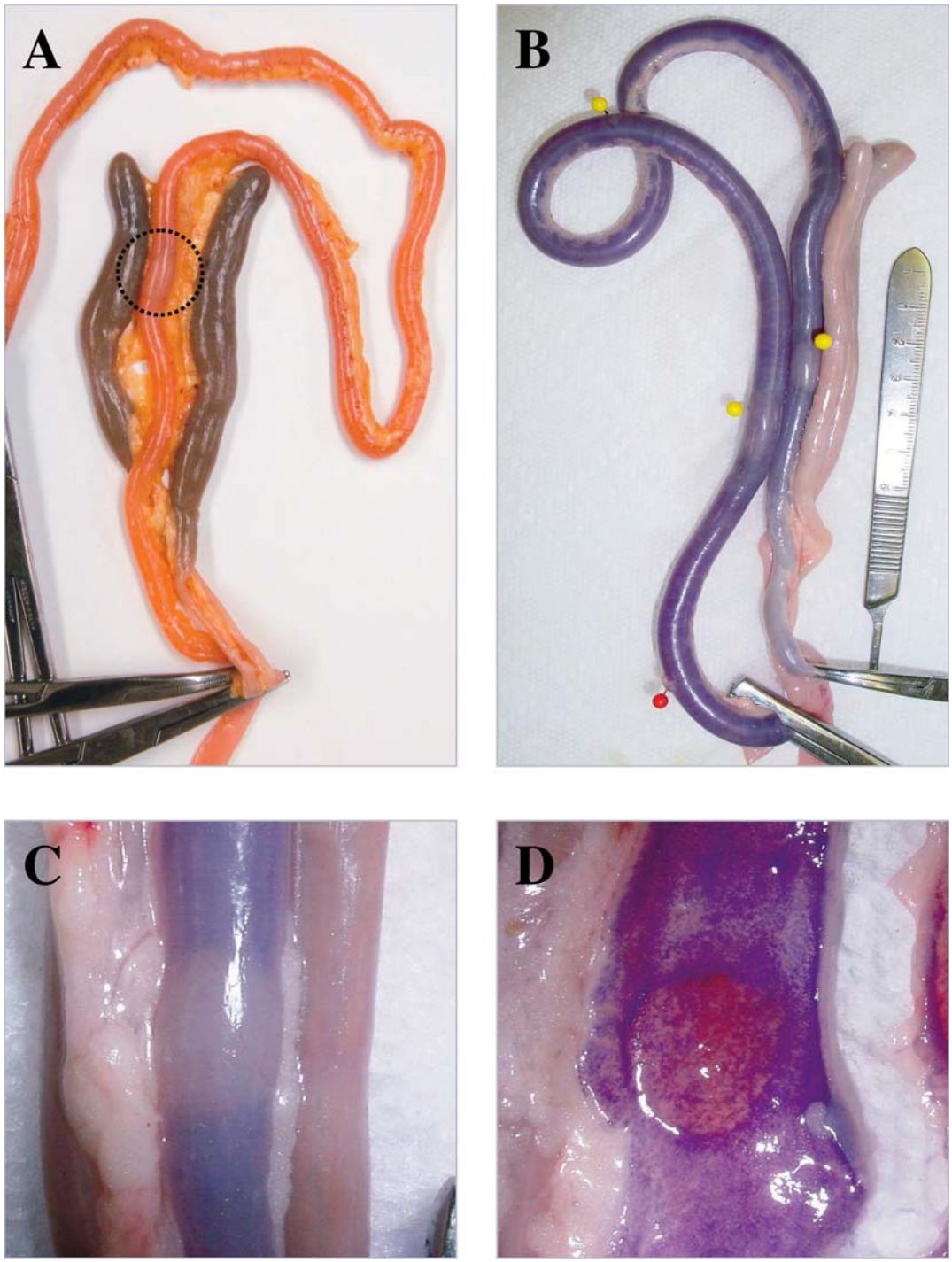

Fig. 1. Gross staining appearance of fresh tissue specimens. (A) Slight enhancement of potential Pp sites with initial infusion of eosin-Y (indicated by encirclement). (B) Distension of GI segment with CV and emergence of presumptive Pp areas (marked by yellow pins). (C) Serosal aspect presentation of a presumptive Pp site as pale pink–white focal area. (D) Mucosal aspect visualization of well-delineated pale pinkish coloration of presumed Pp lymphoid tissue site with contrasting purple gut epithelium.

infiltrations were observed (Fig. 2B). The findings were comparable with the descriptive observations made by Befus *et al.* (1), Burns (3), and Makala *et al.* (18) regarding Pp structure.

Histological assessment of the peripheral tissue, correlated to a region that exhibited mCV purple coloration in fresh tissue, revealed typical morphology characteristic of ileum with no remarkable findings. Numerous villi were observed with columnar epithelium and lamina propria that contained scattered lymphocytes within normal limits (23). The peripheral zone seemed to lack the large organized aggregation of lymphoid tissue.

DISCUSSION

The eosin-Y + mCV staining technique enhanced the gross visualization of Pp lymphoid tissue when applied to fresh GI tract specimens of chickens. Our observations regarding the location and

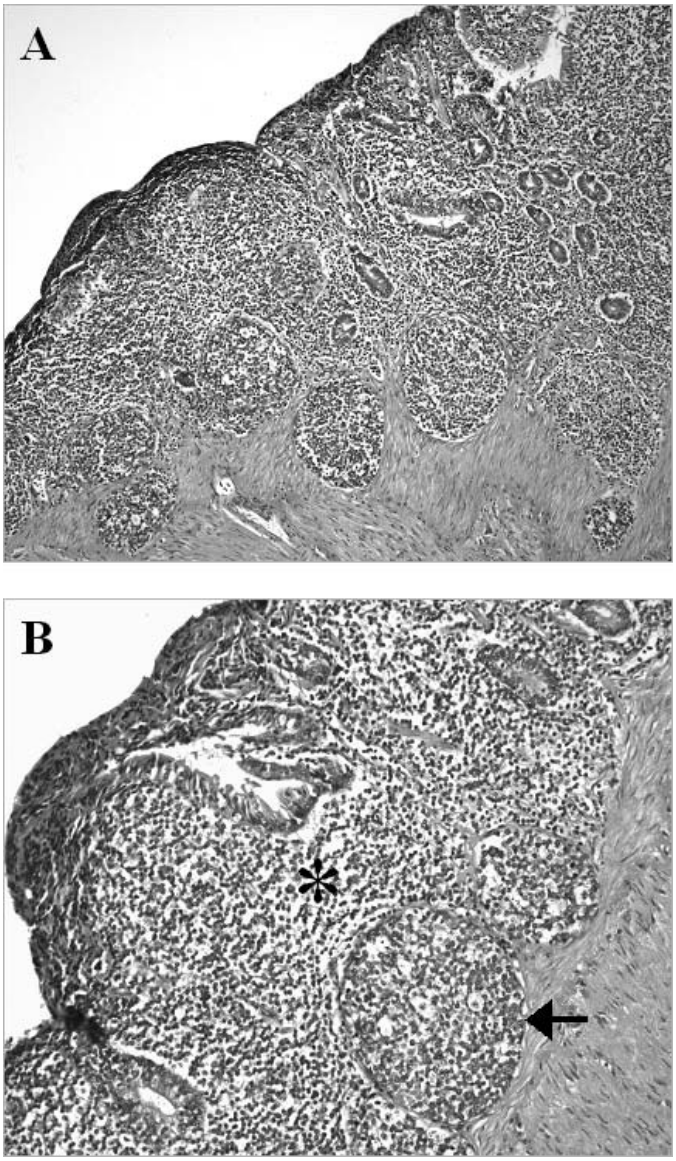

Fig. 2. Microscopy evaluation of H&E-stained slide sections from presumptive Pp sites. (A) Lymphoid aggregations were observed within the lamina propria, with formation of numerous organized lymphoid nodules evident. H&E, 100×. (B) Higher power magnification of a lymphoid follicle (indicated by arrow) and surrounding diffuse interfollicular lymphoid infiltrations (indicated by asterisk). H&E, 200×.

histologic characterization of the distal ileal Pp were similar to those described by Befus *et al.* (1) and Burns (3). Support for our findings of Pp lymphoid tissue existing within both the proximal and distal regions of the ileum, in commercial broilers and SPF White Leghorns, can be derived by comparison of results of Befus *et al.* (1), Burns (3), and Kajiwara *et al.* (12). Development of a distal ileal Pp cranial to the ileocecal junction and one other Pp site in proximity to the Meckel's vitelline diverticulum at days E13–E15 of chick embryogenesis has been demonstrated (12). Thus, these two distinct Pp sites may merit attention as integral GALT involved in chicken GI tract immunity.

The novel staining technique lends an additional and simplified means by which Pp sites can be delineated within unfixed intact intestinal segments. The researcher, when conducting initial screening assessments for Pp location, should no longer be bound to the sole use of methods that demand a substantial portion of time or require a high degree of specialized skill and instrumentation. This new staining procedure can be implemented as a cursory tool for the quick identification of Pp in the chicken. With the application of this novel staining technique, the Pp lymphoid tissue of the chicken would be more readily available for use in studies investigating the mucosal immune responses of the lower alimentary tract.

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